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SUMMARY OF DOCTORAL THESIS

Name: Roxana Yanira Parada Jaco

Title: "A New Host-Specific Toxin, a Protein from Germinating Spores of *Alternaria brassicae* Causing Gray Leaf Spot of *Brassica* Plants"

(*Brassica* 植物黒斑病菌 *Alternaria brassicae* の発芽胞子が生産する新規の宿主特異的蛋白質毒素)

The mechanism for determining specificity in plant-parasite interaction is one of the most intriguing problems in modern plant pathology. Although, in most plants diseases, the factors that determine such specificity are unknown, certain fungal pathogens are known to produce host-specific or host-selective toxins (HSTs) as primary determinants of virulence or pathogenicity. Gray leaf spot caused by *Alternaria brassicae* (Berk.) Sacc., is one of the most important diseases in *Brassica* spp. worldwide. To date, it has been reported that the pathogen produces four phytotoxins named destruxins, and destruxin B, the major phytotoxin is a concentration-dependent HST. However, studies carried out in 2005 in our laboratory, suggested that *A. brassicae* may produce in spore germination fluid (SGF) a toxic compound(s) different from destruxin B, responsible for water-soaked and chlorosis symptoms only on *Brassica* leaves. This study was undertaken with the following objectives: (1) To determine whether destruxin B is involved in infection of the pathogen as a HST, and (2) To isolate and purify the toxic compound(s) released in SGF of *A. brassicae*.

Destruxin B was purified from culture filtrates inoculated with S-193-11 isolate of *A. brassicae*. Analyses by MS and FABMS of the purified fraction matched with data of destruxin B reported previously. The minimum concentration of the toxin required for inducing visible lesion was 50-100 µg/ml on host leaves and 250-500 µg/ml on non-host leaves. The amount of destruxin B released from a spore was 1-2 pg on host (cabbage) and non-host (tomato) leaves and plates after 24 h, and barely increased on tomato leaves and plates after 48 h. The amount released from a spore on cabbage leaves after 48 h was 1.7 times of the amount released after 24 h, in spite of remarkable spore germination and elongation of germ tubes. A similar amount of destruxin B was also found in ungerminated spores and germinated spores. These results indicate that destruxin B, which exists in spores of *A. brassicae*, is released in the suspension before spore germination, but is not released in an enough amount to affect host plants during germination process.

An infection-inducing activity of destruxin B was carried out using a non-pathogenic isolate (O-94) of *A. alternata*. When spores of O-94 suspended in destruxin B at a concentration (100 µg/ml) sufficient for toxicity on host leaves were inoculated on leaves, the spores could not penetrate the host leaves, like that of O-94 spores alone. Interestingly, when spores of O-94 were

suspended in SGF of *A. brassicae* collected from cabbage leaves, the spores were able to invade the host leaves just as the pathogen did. The low molecular weight (LMW) and high molecular weight (HMW) fractions of SGF separated with an ultrafiltration membrane (10 kDa cut off) were tested for toxicity and infection-inducing activity. The toxicity of the LMW fraction with destruxin B was non-specific, and the spores of O-94 suspended in the LMW fraction were not able to invade host leaves. In contrast, the HMW fraction without destruxin B showed host-specific toxicity and allowed colonization of the O-94 spores in the host leaves.

Subsequent investigation was focused on the isolation and purification of the possible new HST(s) detected in HMW fraction. The activity of HMW fraction was abolished by heat and proteinase K treatments, indicating that unlike other toxins reported to be produced by *A. brassicae*, the new compound(s) is a protein(s). A protein toxin was purified from SGF by using ammonium sulfate fractionation and four different chromatography steps. The purified toxin at concentrations of 0.5-1.0 μg/ml induced water-soaked symptoms followed by chlorosis on *Brassica* leaves, while non-host leaves were not affected even at 50 μg/ml, suggesting a host-specific toxicity. The toxin at 0.5-1.0 μg/ml also enabled colonization by a non-pathogenic *A. alternata* to occur only in *Brassica* plants, suggesting that the toxin induces accessibility of host plants to fungal invasion. These data indicate that the toxin from *A. brassicae* fits the criteria of HST and plays a key role as a primary determinant of pathogenicity. Therefore, the toxin was named ABR-toxin as a HST produced by germinating spores of *A. brassicae*. ABR-toxin was heat unstable (60℃ for 15 min) and estimated to be a protein of 27.5 kDa by SDS-PAGE and pI value of the toxin was approximately 7. The N-terminal 21 amino acids were sequenced and FASTA search revealed that it has 81% similarity in 21 amino acid overlap with trypsin precursor (P35049) from *Fusarium oxysporum*. In future research, it will be important to investigate characterization and cloning of a gene encoding ABR-toxin as well as induction mechanism and mode of action of ABR-toxin on host plants.